



AFRL-AFOSR-VA-TR-2017-0076

Mechanism of Stabilization of Labile Compounds by Silk Fibroin Proteins

**David Kaplan
TRUSTEES OF TUFTS COLLEGE INC
169 HOLLAND ST FL 3
SOMERVILLE, MA 21442401**

**04/05/2017
Final Report**

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Air Force Research Laboratory
AF Office Of Scientific Research (AFOSR)/RTB2

REPORT DOCUMENTATION PAGE		Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 05-04-2017		2. REPORT TYPE Final Performance	
		3. DATES COVERED (From - To) 01 Nov 2013 to 31 Oct 2016	
4. TITLE AND SUBTITLE Mechanism of Stabilization of Labile Compounds by Silk Fibroin Proteins		5a. CONTRACT NUMBER	
		5b. GRANT NUMBER FA9550-14-1-0015	
		5c. PROGRAM ELEMENT NUMBER 61102F	
6. AUTHOR(S) David Kaplan		5d. PROJECT NUMBER	
		5e. TASK NUMBER	
		5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) TRUSTEES OF TUFTS COLLEGE INC 169 HOLLAND ST FL 3 SOMERVILLE, MA 01948 US		8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) AF Office of Scientific Research 875 N. Randolph St. Room 3112 Arlington, VA 22203		10. SPONSOR/MONITOR'S ACRONYM(S) AFRL/AFOSR RTB2	
		11. SPONSOR/MONITOR'S REPORT NUMBER(S) AFRL-AFOSR-VA-TR-2017-0076	
12. DISTRIBUTION/AVAILABILITY STATEMENT DISTRIBUTION A: Distribution approved for public release.			
13. SUPPLEMENTARY NOTES			
14. ABSTRACT <p>The objective of this research was to elucidate the fundamental mechanisms by which labile compounds are entrapped and stabilized by silk fibroin protein. The plans built upon our previous studies with silk fibroin for enzyme and antibody stabilization and define the critical interactions between the silk protein matrix and introduced compounds that promote long term stability. An understanding of how the unique chemical and structural features of the silk fibroin stabilize compounds allow for a comparison with more traditional stabilizing agents and shed light on the incompletely understood conditions that lead to stability of labile molecules. During this project, we utilized proteins in blood as a test system to study and understand the role of silk in the stabilization of a range of different analytes, including entrapment, storage and recovery. Here, we successfully used silk fibroin as a solid matrix to encapsulate blood analytes, protecting them from thermally induced damage that can be encountered during transportation or freeze-thaw cycle. We also investigated stabilization of plasma C-reactive protein, a diagnostic biomarker implicated in cardiovascular health, to gain fundamental insight into silk stabilization. These studies represent the first attempt to quantitatively characterize the mechanism controlling the stability of proteins in silk matrices. Furthermore, two screening techniques were utilized to outline excipient concentration ranges that affect silk matrix dynamics. Taken together, the approach herein provides a platform for analyzing silk stabilizing materials, and demonstrates that that rational design of silk formulations based on underlying fundamental mechanisms of stabilization can be used as an approach to optimize stability of encapsulated macromolecules.</p>			
15. SUBJECT TERMS silk			

Standard Form 298 (Rev. 8/98)
Prescribed by ANSI Std. Z39.18

DISTRIBUTION A: Distribution approved for public release.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			ROACH, WILLIAM
Unclassified	Unclassified	Unclassified	UU		19b. TELEPHONE NUMBER (Include area code) 703-588-8302

Mechanism of Stabilization of Labile Compounds by Silk Fibroin Proteins

David L. Kaplan, Tufts University

January 25, 2017 – Final Report

Overview (summary goals for all reports) – The objective is to elucidate the fundamental mechanisms by which labile compounds are entrapped and stabilized by silk fibroin protein. The plans build upon our studies with silk fibroin for enzyme and antibody stabilization and will define the critical interactions between the silk protein matrix and introduced compounds that promote long term stability. Further, an understanding of how the unique chemical and structural features of the silk fibroin stabilize compounds will allow for a comparison with more traditional stabilizing agents and shed light on the incompletely understood conditions that lead to stability of labile molecules. In addition, we plan to complete efforts initiated to refine our understanding of electrogelation mechanisms of silk with regard to differences from pH-induced effects, particularly in flow fields and in adhesion. Finally, we will continue to expand our understanding of silk in terms of structure-function relationships to provide new silk-based materials and new functions for these materials for use in stabilization studies as well as in a broader range of material-related needs.

Silk-Based Stabilization – In a major study, we utilized proteins in blood as a test system to study and understand the role of silk in stabilization of a range of different analytes, including entrapment, storage, recovery (Fig. 1). Advanced diagnostics depend on the availability of high-quality biological samples. These are typically biofluids, such as blood, saliva, or urine, and their collection and storage is critical to obtain reliable results. Without proper temperature regulation protein biomarkers in particular can degrade rapidly in blood samples, an effect that ultimately compromises the quality and reliability of lab tests. Here, we present the use of silk fibroin as a solid matrix to encapsulate blood analytes, protecting them from thermally induced damage that could be encountered during non-refrigerated transportation or freeze-thaw cycles. Blood samples are recovered by simple dissolution of the silk matrix in water. This process is demonstrated to be compatible with a number of immunoassays while providing enhanced sample preservation in comparison to traditional air-drying paper approaches. Additional processing can remediate interactions with conformational structures of the silk protein to further enhance blood stabilization and recovery. This approach can provide expanded utility for remote collection of blood and other biospecimens empowering new modalities of temperature-independent, remote diagnostics. Both research and clinical care often require blood to be collected remote from the laboratory setting. Remote collection presents a logistical and financial challenge, as it requires continuous access to portable cold storage. Although there has been a thrust to develop means to bypass the cold chain, available technologies, such as dried spots, remain ineffective. Specifically, these methods fail to stabilize labile protein biomarkers against thermal damage. Herein we describe an alternative silk matrix encapsulation technique that overcomes these limitations and can be deployed using a simple air-drying approach. Potential clinical and research applications of this technology are far-reaching, and could ultimately decrease hospital burdens, improve patient compliance to monitoring, and open up new testing options for currently underserved populations.

We used a simple method to encapsulate and transport small blood samples for long-term ambient storage and subsequent on-demand recovery and laboratory analysis. Air dried silks provide a protective barrier that physically immobilized blood components with access to minimal residual moisture, in turn conferring conformational stability. We directly demonstrated that the physical entrapment provided by the silk matrix was effective in mitigating thermally-induced degradation and we can infer there are additional benefits such as preventing exposure to enzymatic and UV stresses. Long-term temperature stability of dried silk formats such as films or powders should be useful for field conditions where the silk material was used as the entrapping matrix and recovery of analytes is dependent on sustained solubility, or where a dry transportable format is required prior to mixing with blood. In contrast, liquid plasma demonstrated instabilities above -80°C and after periods of freeze-thaw, consistent with well-established pre-analytical sample management protocol. Indeed, previous reports have indicated that small precipitates can form in plasma isolated from heparinized blood at freezing temperatures above -80°C (for instance the -20°C conditions used herein), and that this is a major contributor to the loss of viability/recoverability of clotting factors in general. Importantly, freeze-thaw damage can be circumvented entirely using the ambient drying approach described here.

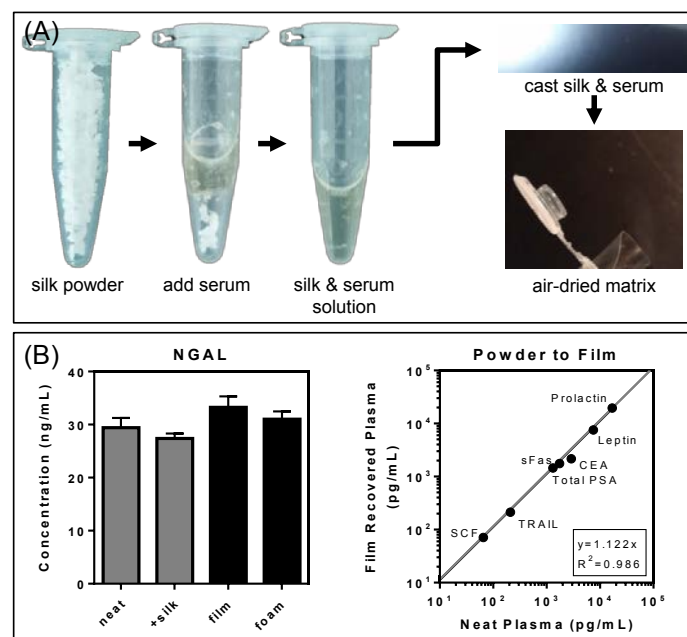


Fig. 1: Silk powder for use as a stabilizing agent in the field. A) A schematic showing the regeneration of silk powder with serum to incorporate stabilizing agent. The formulation is cast and the resulting air-dried matrix placed in an Eppendorf tube. B) (Left) Plasma neutrophil gelatinase-associated lipocalin (NGAL) can be recovered after addition of 16mg silk powder to 50 uL plasma diluted 8x in water (“+silk”), after air-drying the powder/plasma solution (“film”), and also after lyophilizing said the powder/plasma solution (“foam”). Data are average \pm SD of $n=4$ replicate samples from a single donor. (Right) Luminex data demonstrating recovery of 7 biomarkers from a silk powder generated film. Black dots indicate average \pm SD of $n=4$ replicate samples from a single donor. Gray line indicates the best-fit line (equation inset) from linear regression.

In this work we utilized both traditional sandwich ELISA formats as well as bead-based microfluidic immunoassays for detection of the blood-based analytes, demonstrating the adaptability of the silk stabilization approach to a variety of downstream analytical techniques. In principle, the specificity of the immunoassay approach for the blood-based analytes and lack of interaction with silk protein is facilitated by the repetitive and predominately glycine-alanine-serine silk fibroin sequence. The same silk stabilization, recovery, and analysis techniques demonstrated here with blood should be applicable to other biospecimens, including serum, saliva, and urine, as well as most other techniques employed in the analytical lab setting (chemical assays, tandem mass spectrometry, etc.).

During the course of this study we evaluated several formulations or reconstitution alterations in order to improve recovery of analytes in silk-laden materials. In the context of clinical usage, it is likely that several reconstitution buffers will be required per assay panel as dictated by the strength of interactions between analytes and the silk on a case-by-case basis. We anticipate that supplying a few different analyte-specific reconstitution buffers will be well tolerated in industry, assuming there is at least some broader cross-analyte compatibility. Another potential limitation inherent to drying biospecimens – a problem currently addressed in dried blood spot techniques – is that accurate starting volumes of the specimen must be known in order to calculate the final sample dilution upon recovery. This can be addressed by more precisely metering the solution (capillary tube, pipetting, etc.) prior to mixing with the silk, as opposed to simply dabbing a heel or finger prick directly onto the substrate as is currently done in paper. This may add expense but will of course ensure better accuracy. The major focus of the present work was to demonstrate the multifunctional aspects of silk as a sample stabilization and transport system – both mechanical and biological in nature – although traditional pharmaceutical optimization strategies can be employed in future design iterations to enhance the long-term stabilization capacity. Various common excipients and drying techniques are compatible with silk due to the aqueous processing at ambient conditions. The solubility of silk in the presence of other entrapped materials will depend in part on the ratio or stoichiometry of the entrapped biologic to silk, the type of entrapped material, and buffers/excipients/inhibitors typically employed in aiding in stabilization of these biologics. Due to the conformational nature of the protein we also anticipate the compatibility of silk with a variety of commercially available collection devices.

Mechanisms of Stabilization – We have shown that silk fibroin protein has the ability to protect biomacromolecules from thermal degradation, but a deeper understanding of the underlying mechanisms that can fully leverage the stabilizing potential of this matrix has not been realized. We investigated stabilization of plasma C-reactive protein, a diagnostic biomarker implicated in cardiovascular health, to gain fundamental insight into silk stabilizing (Fig. 2 and 3). β -relaxation processes and theory, originally identified with sugar-glass matrices to govern entrapped protein stability, were extended to silk stabilization mechanisms. Specifically, we observed that the addition of antiplasticizing additives that suppress β -relaxation amplitudes in silk matrices resulted in enhanced stability of plasma CRP. These observations suggest fundamental insight into mechanisms, as well as practical strategies to employ with silk protein matrices for enhanced stabilization utility.

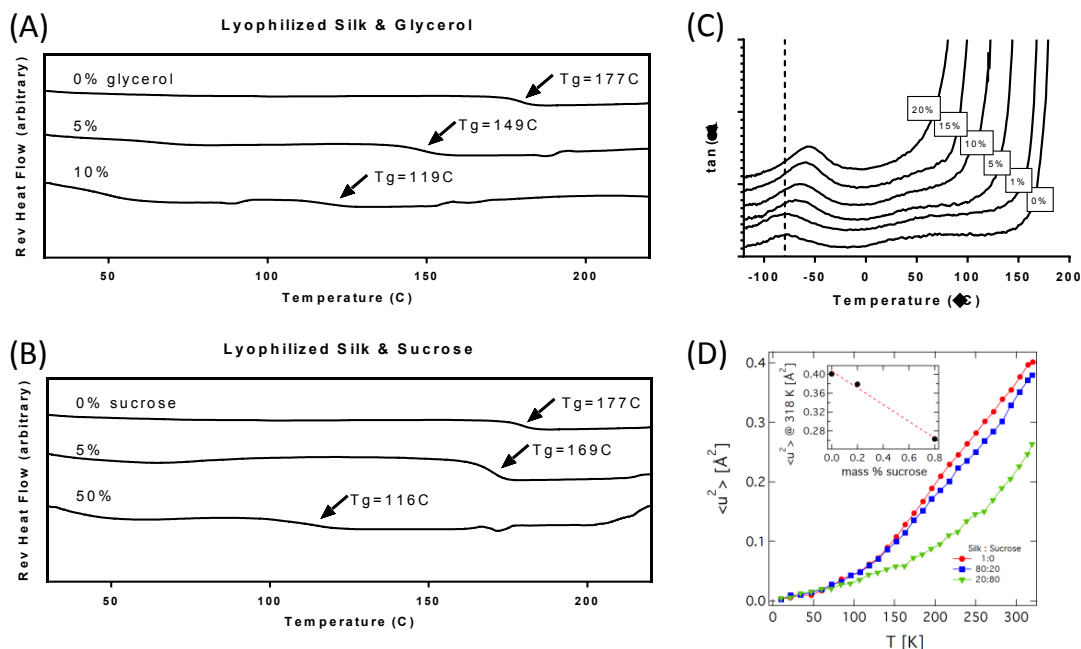


Figure 1: Biophysical analysis of dried silk systems. A) Temperature modulated differential scanning calorimetry of lyophilized silk and glycerol (A) or sucrose (B) cakes. % indicates mass ratio of additive in dried cake. T_g=glass transition temperature. B) Dynamic mechanical thermal analysis (DMTA) of air-dried silk and glycerol films. Dotted line indicates temperature at which the silk alone film undergoes β-relaxation. C) Neutron scattering analysis of silk and sucrose cakes. Inset: mean squared displacement of hydrogen atoms ($\langle u^2 \rangle$) as function of sucrose mass fraction.

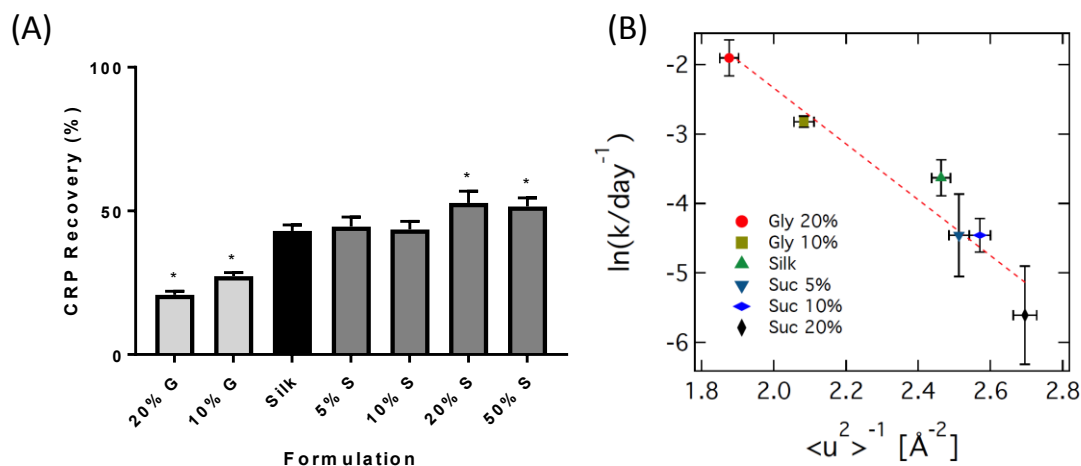


Figure 2: Effect of additives and matrix $\langle u^2 \rangle$ on stability of plasma CRP. A) G=glycerol. S=sucrose. % indicates mass ratio of additive in dried cake. Data are after storage at 45°C for 28 days and normalized to time-matched frozen control. Asterisks indicate significant differences from the silk control formulation at $p < 0.05$ level. B) Plasma CRP degradation rates (k) in silk and glycerol cakes plotted against $\langle u^2 \rangle$ of the encapsulating matrix. Error bars indicate sum of squares error from linear regression.

These studies represent the first attempt to quantitatively characterize the mechanism controlling the stability of proteins in silk matrices. The assumed mechanism is based on work pioneered for predicting protein stability in sugar glasses. In the case of both glycerol and sucrose, protein stability corresponded well to expectations based on neutron scattering data. Furthermore, two screening techniques were utilized to outline excipient concentration ranges that affect silk matrix dynamics. Taken together, the approach herein provides a platform for analyzing silk stabilizing materials, and demonstrates that that rational design of silk formulations based on underlying fundamental mechanisms of stabilization can be used as an approach to optimize stability of encapsulated macromolecules.

Collaborations: (Tufts) *Students:* Meghan McGill, Ben Partlow, Jeannine Colburn; *Faculty:* Peggy Cebe, Physics; Fiorenzo Omenetto, Biomedical Engineering; (outside of Tufts) Rajesh Naik, Patrick Dennis - Air Force; Nancy Kelley-Loughnane - Air Force Materials Lab; Dan Blair/Jeff Urbach - George Washington U.; Vladimir Tsukuk - Georgia Tech; Carole Perry - Nottingham-Trent U.; Greg Holland - San Diego State University; Marcus Cicerone – NIST, Jim Harden - University of Ottawa, Doug Clark - U. California Berkeley